AN ANALYSIS OF THE DYNAMIC FACTORS RESPONSIBLE FOR THE PHENOMENON OF PIGMENT SUPPRESSION IN SALAMANDER LARVAE [1]

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Twitty and Bodenstein (1939 and 1944) and Twitty (1945) have shown that grafted urodele pigment cells which have a relatively rapid developmental rate are able to suppress the differentiation of more slowly developing pro-pigment cells. An examination of the correlation between differences in rates of development and degree of suppression (Lehman, 1950) has demonstrated that a relatively large, though not maximal, difference between the developmental rates of donor and host cells provides the most favorable condition for the suppression of pigmentation. The latter study also showed that although the inhibition of slowly developing cells may be virtually complete in pre-feeding and early larval stages, the effect is not permanent. The more slowly developing melanophores later appear in progressively greater numbers and make a substantial contribution to the final pigment pattern at metamorphosis. There is thus a clear indication that the phenomenon of pigment suppression of the type encountered here [2] should be considered a temporary condition associated with embryonic and early larval periods of pigment differentiation.

The experiments described in this paper are a continuation of the work cited (Lehman, 1950) and attempt to clarify further the developmental mechanics of the suppression phenomenon. To this end, three general aspects of pigment cell behavior have been investigated. Listed in the order treated, they are: A) chromatophore interactions influencing pigment cell migration, B) influences of environmental factors on chromatophore differentiation, and C) intrinsic species differences in pro-pigment cell behavior.

The writer gratefully acknowledges his indebtedness and appreciation to Professor V. C. Twitty, whose personal interest, encouragement, and critical suggestions played a large part in bringing this work to completion.

Materials and Methods

Embryos of five species of salamanders possessing different normal rates of development were used in the following experiments. Proceeding from the most

[1] Based in part upon data included in a dissertation submitted to the Faculty of Stanford University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the School of Biological Sciences.

[2] Not to be confused with the type of pigment inhibition that results from the replacement of anterior trunk neural folds by anterior cranial folds (Niu, 1947). This type of pigment inhibition presents one of the most puzzling enigmas in the entire problem of pigmentation, and is entirely different in character from the suppression effect dealt with in this paper.

rapid to the slowest, this graded series consisted of *Amblystoma tigrinum, A. mexicanum* (the black axolotl), *A. punctatum, Triturus torosus* and *T. rivularis.* The differences in their developmental rates are graphically portrayed in an earlier paper (Lehman, 1950). The embryos of *T. rivularis* were obtained from Robinson creek near Ukiah, California, and those of *T. torosus* were collected from ponds and streams in the vicinity of Stanford University. The eggs of *A. punctatum* were shipped to Stanford University from Connecticut, North Carolina, and Illinois, and those of *A. tigrinum,* from Illinois. *A. mexicanum* eggs were obtained by temperature-induced spawning from axolotls maintained at the Stanford laboratory.

The experiments include the explantation, transplantation, and extirpation of neural folds and neural crest, and the transplantation of epidermis. Unless otherwise stated, experimental embryos along with appropriate controls were reared in individual dishes and kept at 14 to 18° C. The culture methods employed for *in vitro* studies followed the hanging drop technique described by Twitty (1945). The nature of the various graft and explant combinations, along with special details of methods and techniques, are given in conjunction with the description of each experimental series. Developmental age is given in terms of the Harrison stages for *A. punctatum* and *A. tigrinum* and the closely corresponding Twitty and Bodenstein stages for *T. torosus* and *T. rivularis* (see Hamburger, 1942, pp. 202–204, and Rugh, 1948, pp. 94–101). For brevity, single arrows have been used throughout the text to indicate the direction of donor-to-host graft combinations.

Experiments

A. Chromatophore interactions influencing pigment cell migration

Considerable experimental data (Twitty, 1944, 1945, and 1949, and Twitty and Niu, 1948) support the thesis that the primary motivating force in pigment cell migration is intrinsic to the chromatophore population itself, rather than being due to chemotactic (Rosin, 1943, and Holtfreter, 1947) or thigmotactic (Weiss, 1945, and Dalton, 1949 and 1950) responses to the tissue environments the cells may enter. The movement of pigment cells can be considered the expression of intercellular antagonisms (in the sense of Holtfreter's "negative tissue affinities" (1939)), which cause the cells to repel one another mutually and, in a manner of speaking, to strive for isolation. Twitty (1945) has shown that the same intercellular antagonisms that are responsible for migration also enable pigment cells to impede the migration of other melanophores that might attempt to invade an occupied terrain. The experiments in Series 1, 2, and 3 were carried out in order to discover the duration of the period in which pigment cells manifest mutual antagonisms.

Series 1. Duration of mutual antagonisms between chromatophores in vitro: Cultures of trunk neural crest and neural folds were prepared in the following manner. Mesoderm-free neural crest, along with a small amount of underlying neural tissue, was removed from Stage 23–25 tail bud embryos after the mid-dorsal epidermis had been carefully stripped away. Mesoderm-free trunk neural folds were taken from Stage 16–17 neurulae. The strips of excised neural crest and neural fold were divided into three parts of equal size and each piece was then isolated in a drop of Holtfreter solution on a cover glass. The cultures were sealed

PLATE I

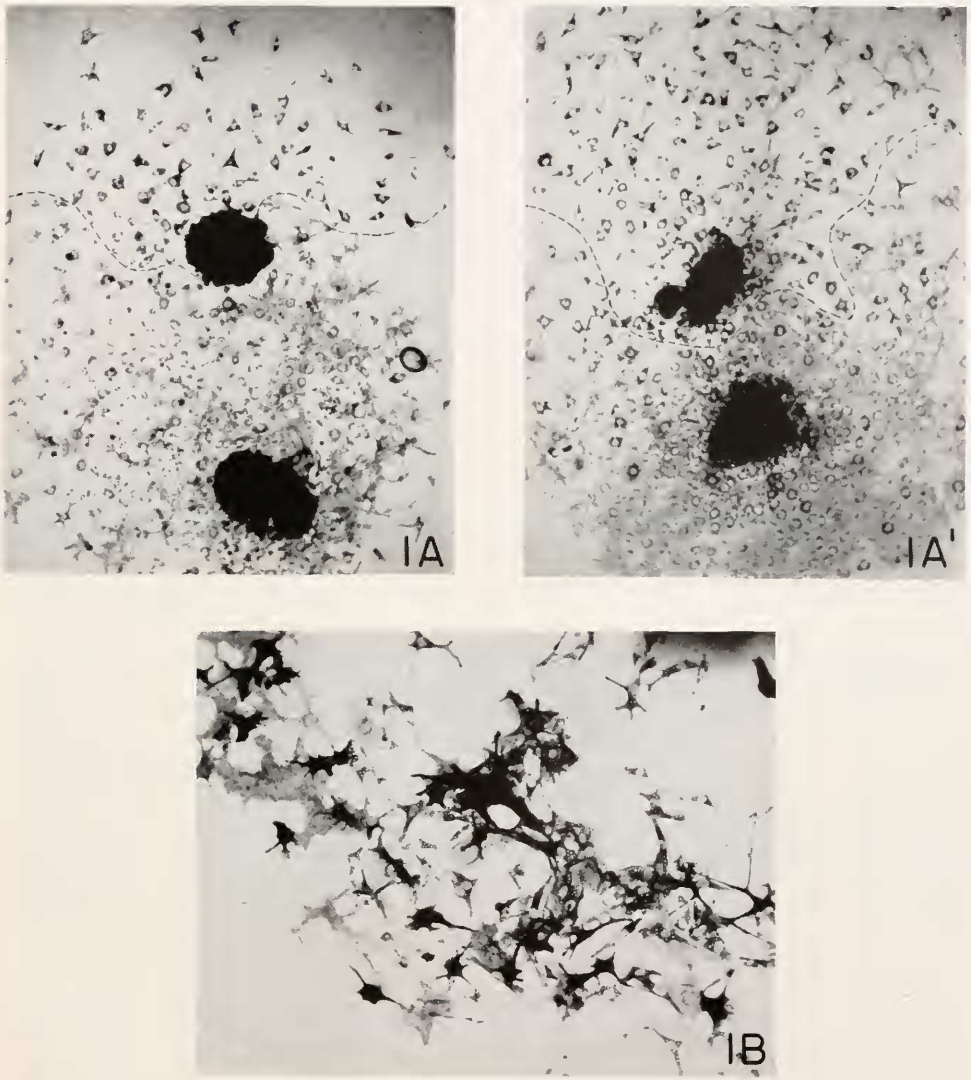


FIGURE 1A. A culture in Holtfreter solution of two pieces of neural crest differing moderately in age, showing a primary outgrowth (below) of *A. punctatum* chromatophores 11 days after explantation, and a secondary outgrowth (above) of *T. torosus* pro-pigment cells heavily charged with yolk platelets 5 days after explantation. A broken line marks the approximate boundary between the cells of the two outgrowths.

FIGURE 1A'. A different culture from the same series as figure 1A, 3 days later.

FIGURE 1B. A culture of two pieces of *T. torosus* neural crest of widely different age. Fully differentiated melanophores and light gray xanthophores 16 days after explantation can be seen with many pro-pigment cells of the secondary explant (6 days after isolation) migrating over them.

in inverted depression slides and three to four days were allowed for attachment of explants before the slides were righted and examined. The cultures were kept at 18° C. for the duration of the experiment.

The tendency for cells to migrate *in vitro* was taken as the indication of the presence of intercellular antagonisms. Using this basis for judgment, it appears that the initial development of pronounced intercellular antagonisms coincides with the onset of normal migration. The basis for this assumption is found in the commonly observed fact that a longer period of time was required for explanted neural folds to give outgrowths than was needed by neural crest. In either case, outgrowths in cultures did not begin until the explants reached an age approximating that at which pro-pigment cells normally began to leave the neural crest in control embryos. According to Detwiler (1937), this corresponds to the period between Stages 28 and 31 for *A. punctatum*, and it is assumed that approximately the same holds true in the other species used.

A simple modification of an experiment devised by Twitty (1945) was used in attempting to discover the duration of the period in which pigment cells actively repel one another. Instead of placing two explants of neural crest of the same age in a single drop of medium, pieces of neural fold were introduced into hanging drops of Holtfreter solution which possessed a previously explanted fragment of neural crest with its established outgrowth of cells (hereafter referred to as the "primary outgrowth"). By means of glass needles, the younger "secondary" explant was moved as near as possible to the edge of the primary outgrowth. The fluid in the drop was replenished before being re-sealed in an inverted depression slide. The cultures were not disturbed for three or four days to permit the secondary explants to become firmly attached to the glass surface. From 24 to 70 preparations of each of the following double explant combinations were made: *T. torosus* on *T. torosus*, *T. torosus* on *A. punctatum*, *A. punctatum* on *A. punctatum*, and *T. rivularis* on *A. punctatum*. In some cases the primary outgrowths were relatively young, and in others they were in advanced stages of differentiation when the secondary explant was added.

The results of Series 1 can be briefly summarized by saying that when the primary and secondary outgrowths differed only moderately in age (*i.e.*, 4 to 8 days), there was little mixing of cells when the margins of young and old outgrowths came in contact with one another. This is illustrated in Figures 1A and 1A′. The opaque areas in the figures show the positions of the explants (primary below, secondary above), and the broken line in each figure marks the approximate boundary between cells of the primary and secondary outgrowths. It will be noted that cells of the latter have migrated extensively only in previously unoccupied areas. This is taken as evidence for the existence of intercellular antagonisms which enable the partially differentiated cells of the primary outgrowth to halt the advance of younger cells. Fully mature chromatophores, on the other hand, offered virtually no impediment to the migration of young cells. This can be seen in Figure 1B in which young cells, abundantly supplied with yolk platelets, have migrated freely over the heavily pigmented cells of the primary outgrowth.

There is thus a clear indication that, *in vitro* at least, incompletely differentiated melanophores offered a more effective barrier to the migration of young pro-pigment cells than was presented by mature melanophores. More striking confirmation of this generalization was provided in the following series of transplantations.

Series 2. Duration of mutual antagonisms between chromatophores in vivo: In order to discover whether the age and distribution of older neural crest cells would influence the migration of younger cells within the embryo, three series of transplantations were carried out. They involve the grafting of identical pieces of Stage 23 *A. punctatum* trunk neural crest unilaterally onto the flank at the yolk border of Stage 24, 32, and 37 + *T. rivularis* embryos.

Series 2a. Stage 23 *A. punctatum* neural crest grafted onto the flank of Stage 24 *T. rivularis* hosts: At the time of operation, both donor and host pro-pigment cells were at essentially the same developmental age. However, owing to the intrinsically slow pace of *T. rivularis* development, the host cells subsequently lagged considerably behind those of donor origin. Donor cells spread radially from the graft and achieved a wide distribution over the yolk mass. They appropriated areas anterior and posterior to the graft and extended as far dorsally as the base of the fin (Fig. 2A). Host cells were prevented from making an appearance in these areas until after Stage 41.

PLATE II

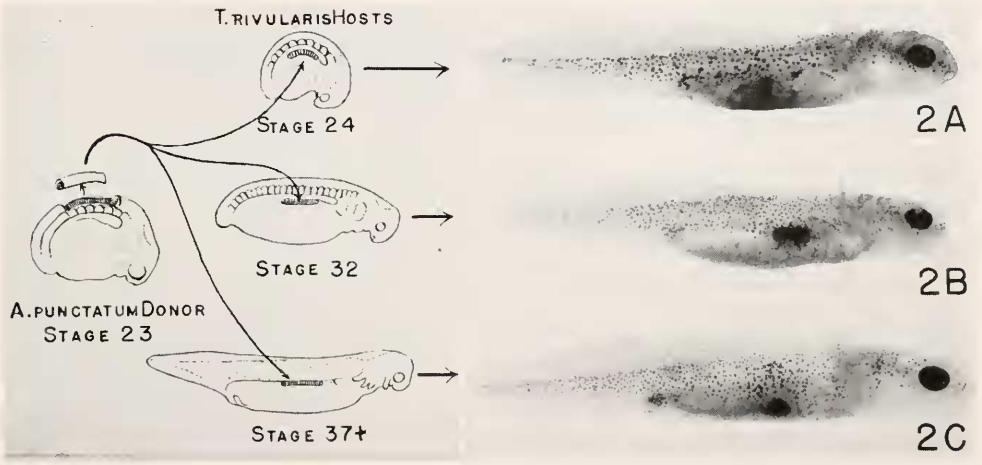


FIGURE 2A. Stage 23 *A. punctatum* neural crest grafted at the yolk border of *T. rivularis* host at Stage 24; figure 18 days after operation, at Stage 44 +.

FIGURE 2B. Stage 23 *A. punctatum* neural crest grafted at the yolk border of *T. rivularis* host at Stage 32; figure 8 days after operation, at Stage 42.

FIGURE 2C. Stage 23 *A. punctatum* neural crest grafted at the yolk border of *T. rivularis* host at Stage 37 +; figure 11 days after operation, at Stage 45. See explanation in text.

Series 2b. Stage 23 *A. punctatum* neural crest grafted onto the flank of Stage 32 *T. rivularis* hosts: At the time of operation, host cells were in an early stage of active migration over the flanks, but as yet were not pigmented. By host Stage 36, a full complement of *T. rivularis* host cells was faintly visible on the flank dorsal to the graft before any donor melanophores appeared. When the latter did make an appearance, their distribution was restricted to the immediate vicinity of the transplant and to the surface of the yolk mass ventrolateral to the graft site (Fig. 2B). These results are noteworthy, inasmuch as they clearly demonstrate that, if given a moderate advantage in age, *T. rivularis* melanophores are able to inhibit the

migration of *A. punctatum* pro-pigment cells as effectively as they themselves were inhibited by *A. punctatum* cells in Series 2a.

Series 2c. Stage 23 *A. punctatum* neural crest grafted onto the flank of Stage 37 + *T. rivularis* hosts: The pigmentation of host melanophores was well advanced at the time *A. punctatum* neural crest was grafted on the flank. Donor pigment cells were first observed at host Stage 40 − and, as can be seen in Figure 2C, they were able to migrate radially in all directions from the transplant. This result obtained in spite of the presence of fully mature host cells dorsal to the graft. Therefore, although the distance of migration was less extensive, the distribution of donor melanophores was similar to that achieved by *A. punctatum* cells in Series 2a.

The results from transplantations in Series 2 are in complete accord with those obtained from double explantations in Series 1. Series 2a and 2b duplicate *in vivo* the results obtained in Series 1 cultures in which the two explants differed only moderately in age and exhibited strong mutual antagonisms between cells of opposing outgrowths. Series 2c, on the other hand, is comparable to those explantations in which the fully differentiated cells of the primary outgrowth offered little opposition to invasion by young pro-pigment cells.

Series 3. Experimental modification of the degree of pigment suppression obtained in chimeric-crest combinations: Additional evidence concerning the relationship of intercellular antagonism to pigment suppression was obtained by modifying the standard procedure in preparing "chimeric-crest embryos" (*i.e.*, embryos in which one trunk neural fold was replaced at Stage 17 by a corresponding fold from another species; as a consequence, one side of the resultant neural crest is of donor and the other of host origin; see Lehman, 1950).

Series 3a. Development of *A. mexicanum* → *T. torosus* chimeric-crest embryos at low temperature: This chimeric-crest combination was selected because it had been shown (Lehman, 1950) that *A. mexicanum* chromatophores (which possess a developmental rate intermediate between those of *A. tigrinum* and *A. punctatum*) were most effective in suppressing the differentiation of Triturus melanophores. By subjecting *A. mexicanum* → *T. torosus* chimeric-crest embryos to low temperature following the operation, it was found that the high degree of inhibitory action of *A. mexicanum* chromatophores could be greatly minimized. Embryos kept for 30 days at 6° C. had advanced from Stage 17 to Stage 29–30 when they were finally returned to standard temperatures (14 to 18° C.). The degree to which *T. torosus* pigmentation was suppressed following this prolonged and severe chilling was markedly reduced. Figure 3A shows a normal *T. torosus* larva and Figure 3A′ shows a cold treated *A. mexicanum* → *T. torosus* chimeric-crest host. It will be noted that *T. torosus* melanophores on the experimental animal are only slightly fewer in number than those on the control. The contrast between these results and those obtained in chimeric-crest larvae reared at standard temperatures is evident by comparing Figures 3A′ and 3A″. In the latter, many more *A. mexicanum* pigment cells have differentiated and the suppression of *T. torosus* melanophores throughout the trunk region is essentially complete. This result obtained in spite of the presence of *T. torosus* neural crest along the entire anteroposterior axis of the host embryo.

This experiment demonstrates that the degree of suppression in chimeric-crest larvae is subject to modification by special treatment. However, it is not known

whether cold treatment resulted in an increase or a decrease in the difference between donor and host developmental rates. It is possible that the two components of the chimeric-crest were affected unequally or were retarded to such a degree that the normal disparity in rates of development was, in effect, eliminated or altered sufficiently to permit partial *T. torosus* pigmentation.

Series 3b. Chimeric-crest transplantations involving donor and host neurulae of different developmental age: The *A. punctatum* → *T. rivularis* combination was selected as offering the most favorable opportunity for observing the effects of slight alterations in the disparity between normal donor-host developmental rates. The difference between the developmental rates of these two species is relatively large, but has been shown (Lehman, 1950) to be slightly sub-optimal for obtaining a maximal degree of suppression. In order to reduce the difference between the developmental rates of donor and host tissues as much as possible, a trunk neural fold from young (Stage 14 −) *A. punctatum* neurulae was grafted in substitution for a neural fold on *T. rivularis* hosts in which the neural folds were just beginning to fuse (Stage 19 +). It was thought that by this means a sufficient age handicap might be imposed upon the grafted *A. punctatum* cells to reduce significantly their capacity to suppress *T. rivularis* pigmentation. The results are illustrated in Figure 3B′. It will be noted that *T. rivularis* melanophores are scattered abundantly over the flanks and fin. The more intensely pigmented *A. punctatum* melanophores are, in the main, aggregated ventrolaterally near the yolk border. Compare this distribution of donor and host cells with that in Figure 3B″, which is typical of *A. punctatum* → *T. rivularis* chimeric-crest larvae in which donor and host embryos were at the same developmental age (Stage 17) at the time of operation. The unusual arrangement of pigment cells shown in Figure 3B′ can be accounted for by assuming that *A. punctatum* cells were the first to migrate from the neural crest but were very shortly followed by host pro-pigment cells. The second wave, composed of *T. rivularis* cells, may be visualized as exerting mutual antagonisms which not only resulted in their own dispersion, but also kept the *A. punctatum* cells advancing peripherally until pigmentation took place and migration stopped.

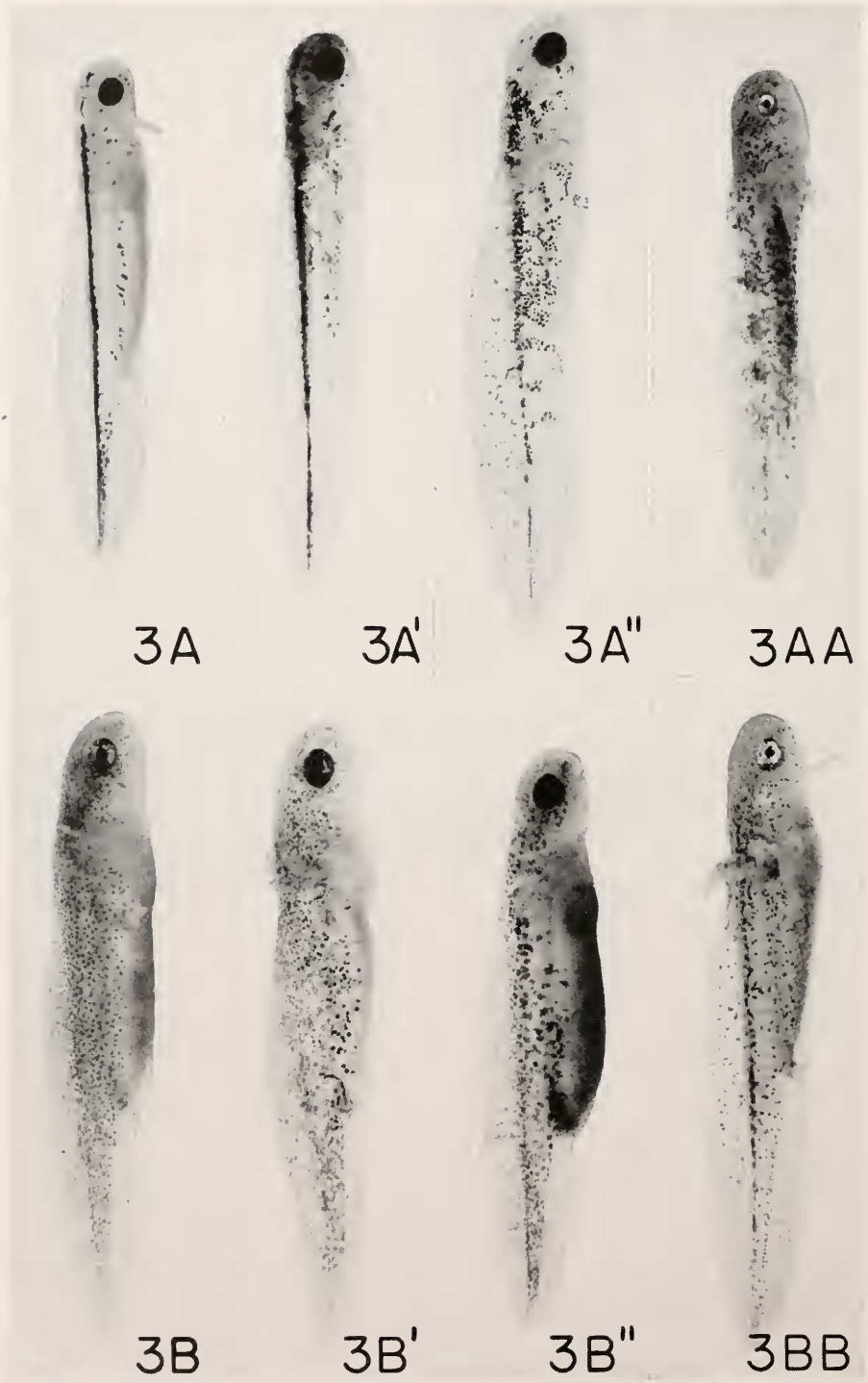
The experimental evidence provided by Series 1, 2, and 3 indicates that the period during which developing chromatophores exhibit antagonistic properties begins with the onset of migration, is most pronounced while the cells are becoming pigmented, and declines as melanophores approach complete histological differentiation. However, since they lose this character as they mature, why is it that in some Amblystoma—Triturus chimeric-crest combinations the suppression effect persisted long after the Amblystoma cells were fully pigmented?[3] The analysis of the factors responsible for this "secondary" delay in the differentiation of Triturus melanophores is the primary concern of the next section.

B. Influence of environmental factors on chromatophore differentiation

Many experiments have revealed that the ability of pro-pigment cells to synthesize melanin is in a large measure governed by the tissues surrounding them

[3] *T. torosus* melanophores, if failing to differentiate during the normal period before Stage 41, did not appear until the 18–20 mm. larval stage; *T. rivularis* melanophores, conversely, began to appear in Amblystoma pigment areas as early as Stages 41 to 44 and became progressively more abundant as development proceeded (Lehman, 1950).

PLATE III



(see reviews by DuShane, 1943 and 1948; Rawles, 1948; Twitty, 1949; and Lerner and Fitzpatrick, 1950). The precise nature of the environmental contribution to pigmentation is problematical; however, the most prevalent suggestion is that the epidermis and mesoderm supply hypothetical substances that act either as precursors of melanin or have some enzymatic action affecting the production of this pigment. An alternate theory has recently been advanced (Dalton, 1949 and 1950) to account for differences between white and black axolotls; it suggests that "the primary inhibitory action of white tissues was not on melanin synthesis but on the migration of pro-pigment cells" (1950, p. 152). Since the general applicability of Dalton's "migration-inhibition hypothesis" is still conjectural, the terminology of the more familiar "precursor-enzyme" hypothesis will be used in the following. The term "melanogenic substances" will be used to designate materials of unidentified nature and action which foster melanogenesis.

The problem at hand concerns the question raised at the close of the preceding section: namely, what is the basis for the secondary delay in Triturus melanophore differentiation after the pigmentation of Amblystoma cells was apparently complete on chimeric-crest embryos (for specific description, see Lehman, 1950)? If the "suppressed" Triturus cells were able to migrate onto the flanks after the fully pigmented Amblystoma cells had lost their antagonistic character, the secondary delay might be explained by: 1) a decline in the capacity of older epidermis to promote pigmentation, and/or 2) the depletion of melanogenic substances to subthreshold levels by Amblystoma cells which pre-empted available terrains at an earlier time. In either case, the question is reduced to whether or not the availability of melanogenic materials was associated with the secondary delay in pigmentation after intercellular antagonism could no longer be effective in suppressing the differentiation of these cells.

Series 4. Explantation of A. mexicanum → T. torosus chimeric-crest in peritoneal fluid: In an attempt to throw light on the above question, neural crest from *A. mexicanum → T. torosus* chimeric-crest embryos was cultured in peritoneal fluid obtained by abdominal puncture from spawning female salamanders. This culture medium was used, inasmuch as Twitty and Bodenstein (1939) had shown it to be more effective in promoting pigmentation *in vitro* than physiological salt solutions. It was reasoned that if melanogen concentration played any part in secondarily delaying pigmentation, it should be possible to induce some *T. torosus* cells to become pigmented by providing a rich melanogenic environment for their

PLATE III

FIGURE 3A. Normal pre-feeding *T. torosus* larva at Stage 40+.

FIGURE 3A′. Cold treated *A. mexicanum → T. torosus* chimeric-crest host at Stage 42 (explanation in text).

FIGURE 3A″. Stage 45 *A. mexicanum → T. torosus* chimeric-crest host reared at 14 to 18° C.

FIGURE 3AA. Normal pre-feeding *A. mexicanum* larva at Stage 41+.

FIGURE 3B. Normal pre-feeding *T. rivularis* larva at Stage 42.

FIGURE 3B′. Stage 14 − *A. punctatum* → Stage 19+ *T. rivularis* chimeric-crest host; figured at Stage 42 (explanation in text).

FIGURE 3B″. *A. punctatum → T. rivularis* chimeric-crest host in which both donor and host embryos were at Stage 17 at time of operation; figured at Stage 41.

FIGURE 3BB. Normal pre-feeding *A. punctatum* larva at Stage 44+.

development. The value of using this particular chimeric-crest combination is apparent from the fact that *in vivo* none, or at best very few, of the *T. torosus* propigment cells would have differentiated before the 18–20 mm. larval stage (Lehman, 1950).

The chimeric-crest embryos which provided neural crest for this experiment were prepared in the usual manner. After the orthotopically grafted *A. mexicanum* trunk neural fold had healed in place and neurulation was complete, the dorsal epidermis was stripped away. The exposed chimeric-crest was removed and divided into three pieces of equal size. Forty-three pieces were isolated in drops of

PLATE IV

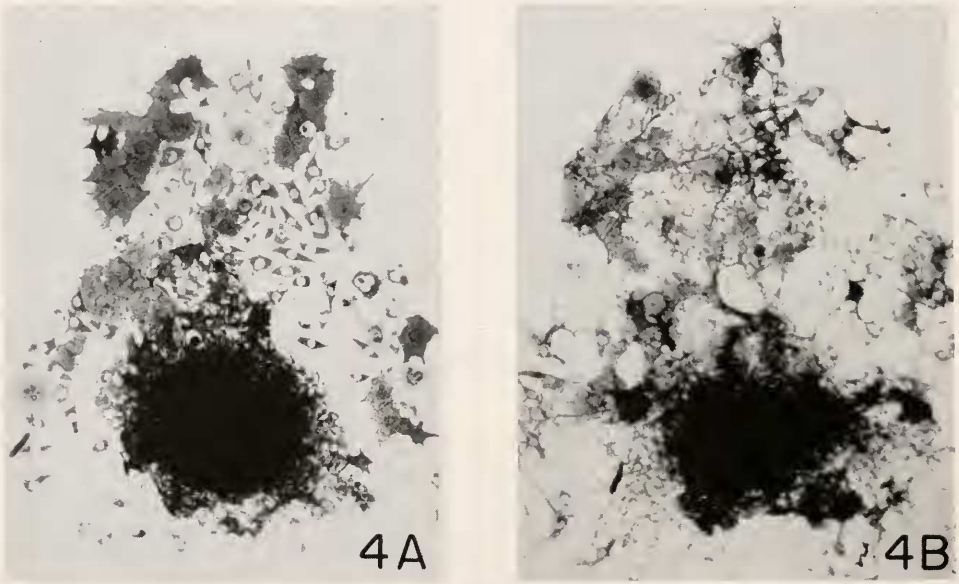


FIGURE 4A. A culture of *A. mexicanum* → *T. torosus* chimeric-crest in peritoneal fluid; figured 4 days after explantation. Well-differentiated *A. mexicanum* melanophores and xanthophores are peripherally located in the outgrowth. Small spindle shaped *T. torosus* propigment cells are easily recognized by their large yolk reserves.

FIGURE 4B. The same culture shown in figure 4A seven days after isolation. *T. torosus* cells are partially pigmented.

peritoneal fluid and kept at 18° C. for the duration of the experiment. After three days, outgrowths consisting of both *A. mexicanum* and *T. torosus* cells were well established. The *A. mexicanum* cells were for the most part located peripherally, thereby indicating that they had probably migrated from the explant in advance of the *T. torosus* cells. Figure 4A shows a four day culture in which *A. mexicanum* chromatophores are already well differentiated, whereas *T. torosus* cells are as yet unpigmented. In this photograph, small spindle shaped *T. torosus* cells appear dark, owing to the bi-refringence of numerous yolk platelets in the cytoplasm. *A. mexicanum* pigment cells are readily distinguishable from them by their larger size and smaller yolk reserves. Figure 4B shows the same culture three days later

after the *T. torosus* cells were partially pigmented. They later became fully differentiated but are not figured, since that by that time the majority of *A. mexicanum* cells had either become punctate or had detached themselves from the glass surface and presented a poor photographic subject.

One can conclude from Series 4 that the temporary association of Triturus and Amblystoma pro-pigment cells in chimeric-crest combinations does not significantly diminish the capacity of Triturus melanophores to migrate from the neural crest. Nor does it prevent their becoming pigmented *in vitro* when an excess of melanogenic material is available.

Series 5. Transplantation of Amblystoma epidermis on T. torosus chimeric-crest host: Amblystoma epidermis, which is more strongly melanogenic than that of *T. torosus* (DeLanney, 1941), was grafted on the flanks of chimeric-crest *T. torosus* hosts. This was done in an attempt to improve the regional conditions for pigmentation to such a degree that latent *T. torosus* melanophores, if present in the area, would become pigmented. It should be pointed out that early in their development *T. torosus* melanophores are uniformly distributed over the flanks and later re-aggregate along the dorsal border of the somites (Figs. 3A and 5A) as a response to the region of highest melanogenic activity in the embryo (Twitty, 1945). In this experiment, advantage was taken of the fact that the dorsal re-aggregation can be prevented if melanogenic conditions are sufficiently improved elsewhere as, for example, under grafted Amblystoma epidermis.

Series 5a. Orthotopic transplantation of Stage 27 *A. punctatum* flank epidermis on Stage 27 *A. punctatum* → *T. torosus* chimeric-crest embryos: The suppression phenomen was in general poorly expressed in this chimeric-crest combination and an appreciable number of host cells were regularly identifiable along the dorsal somite border (Fig. 5A′). It was therefore not surprising to find that host cells also appeared on the flanks at the site of epidermal grafts. One case from this series is shown in Figure 5A′, in which the location of the flank graft has been marked by a broken line. Most of the host cells within the area are in the contracted phase and appear as small dense points. This condition is not uncommon when differentiation takes place over the yolk mass as has occurred here. This experiment does not give any indication as to whether the *T. torosus* melanophores under the epidermal grafts represent, 1) latent melanophores which otherwise would have been suppressed, or 2) pro-pigment cells which merely failed to undergo secondary retraction to the dorsal somite border. Although no additional information is available, it is thought that the second alternative is probably correct.

Series 5b. Orthotopic transplantation of Stage 27 *A. mexicanum* flank epidermis on Stage 27 *A. mexicanum* → *T. torosus* chimeric-crest embryos: The strong melanogenic character of *A. mexicanum* epidermis was evidenced by a pronounced increment in the number of donor melanophores in the graft areas (Figs. 5B and 5B′). *T. torosus* pigment cells were not identified in the graft region except in those instances in which the transplanted epidermis extended dorsally beyond the base of the fin. In the latter case (Fig. 5B″), a few *T. torosus* melanophores frequently were observed under the grafted epidermis along the dorsal border of the somites. The ability of *A. mexicanum* epidermis to foster melanization in *T. torosus* cells *only* near the mid-dorsal line is interpreted to mean that *A. mexicanum* melanophores probably prevented the outgrowth of *T. torosus* pro-pigment cells

until early pre-feeding larval stages. If this were not the case, it would be difficult to account for the failure of *T. torosus* melanophores to respond to the strong melanogenic influence of the grafted epidermis as was the case in Series 5a.

It is probably safe to assume that availability of melanogenic substances was involved in the secondary delay in the differentiation of host cells in *A. mexicanum* → *T. torosus* chimeric-crest larvae, since some *T. torosus* melanophores did become pigmented *in vitro* (Series 4) and *in vivo* (Series 5b) when provided with a rich melanogenic environment.

Series 6. Transplantation of young and old ventral epidermis mid-dorsally on T. torosus tail-bud embryos: It was suspected that the temporal decline in the melanogenic strength of *T. torosus* epidermis described by Twitty (1936) might also contribute to the secondary delay in pigmentation of *T. torosus* cells in chimeric-crest embryos. With this in mind, Twitty's experiments were repeated for the purpose of determining the stage at which the melanogenic activity of the epidermis falls to subthreshold levels for *T. torosus* pigmentation.

The dorsal epidermis covering the neural tube and mesodermal somites was removed from the mid-trunk region of early *T. torosus* tail-bud embryos. Special care was taken to prevent injury to the underlying neural crest and mesoderm. The region of extirpation was then covered by mesoderm-free epidermis taken from the belly region of *T. torosus* donors of graded age. Belly epidermis was used in order to minimize the likelihood of transferring pigment cells adhering to donor epidermis. This region is not only farthest from the neural crest, but also Twitty and Bodenstein (1939) and DeLanney (1941) have shown that a barrier of unidentified character for a time prevents the ventral migration of pigment cells below the yolk border. The graft site was readily identifiable in larval stages, owing to the failure of grafted belly epidermis to respond to the dorsal fin inductor (Figs. 6A and 6B). Three classes of combinations were prepared; each consisted of 12 experimental animals, along with appropriate donor and host controls. The developmental age of donor controls was recorded when the hosts reached Stage 35, which coincides with the first appearance of host melanophores on the flank. This was done for the purpose of establishing an index of the age difference between donor and host epidermis during the active period of host melanophore differentiation.

Series 6a. Stage 13 + *T. torosus* belly epidermis grafted on Stage 28 *T. torosus* tail-bud hosts: Three days following the operation at host Stage 35, pigment cells began to appear in donor and host areas. At this time, donor controls had progressed to Stage 30 +. Thereafter, host pigment cells continued to develop in an entirely normal manner and formed dorsal bands which were essentially similar to those at non-graft levels and on host controls (Fig. 6A). The competence of young epidermis to foster pigmentation is apparently as great as that of the slightly older host epidermis.

Series 6b. Stage 33 *T. torosus* belly epidermis grafted on Stage 23 *T. Torosus* tail-bud hosts: In the second series, four days were required for the Stage 23 hosts to advance to Stage 35. During this interval, donor controls had progressed to Stage 38. Fewer melanophores per unit area became pigmented in the graft region than under host epidermis, but those which did appear were as darkly pig-

mented as those in non-graft areas. These results suggest that shortly after Stage 38, there is a decline in the melanogenic strength of *T. torosus* epidermis.

Series 6c. Stage 37 + *T. torosus* belly epidermis grafted on Stage 23 *T. torosus* tail-bud hosts: By the fourth post-operative day, pigment cells were visible on the Stage 35 host. By this time the donor controls had advanced to Stage 40 +. The number of melanophores that differentiated in graft areas was very drastically reduced and there were several instances in which their elimination was almost complete. This condition is shown in Figure 6B, in which the graft region has been outlined by a broken line. It should be mentioned that technical difficulties were encountered in transplanting Stage 37 + epidermis which has a strong tendency to curl. Healing was also slow and some of the host neural crest was lost before the wound was completely closed. It is possible that this may have caused a moderate reduction in the number of pigment cells in the graft region, but it is improbable that this factor could alone be responsible for the results obtained. Evidence for the presence of latent melanophores at graft levels was provided, moreover, by the presence of normal numbers of fully differentiated pigment cells around the neural tube, even though they were lacking under the overlying grafted skin. Melanophores around the neural tube are faintly visible through the myotomes in Figure 6B.

One can tentatively conclude that between Stages 38 and 40 + or shortly thereafter, *T. torosus* epidermis to a large measure loses its capacity to promote pigmentation in prospective *T. torosus* melanophores.[4] Thus, if rapidly developing Amblystoma pigment cells were able to delay the differentiation of *T. torosus* cells until Stage 41 (that is, by intercellular antagonisms that prevented outgrowth or by reducing melanogens to sub-threshold levels), then ageing of the *T. torosus* epidermis itself could become a significant factor contributing to a secondary delay in the pigmentation of *T. torosus* melanophores.

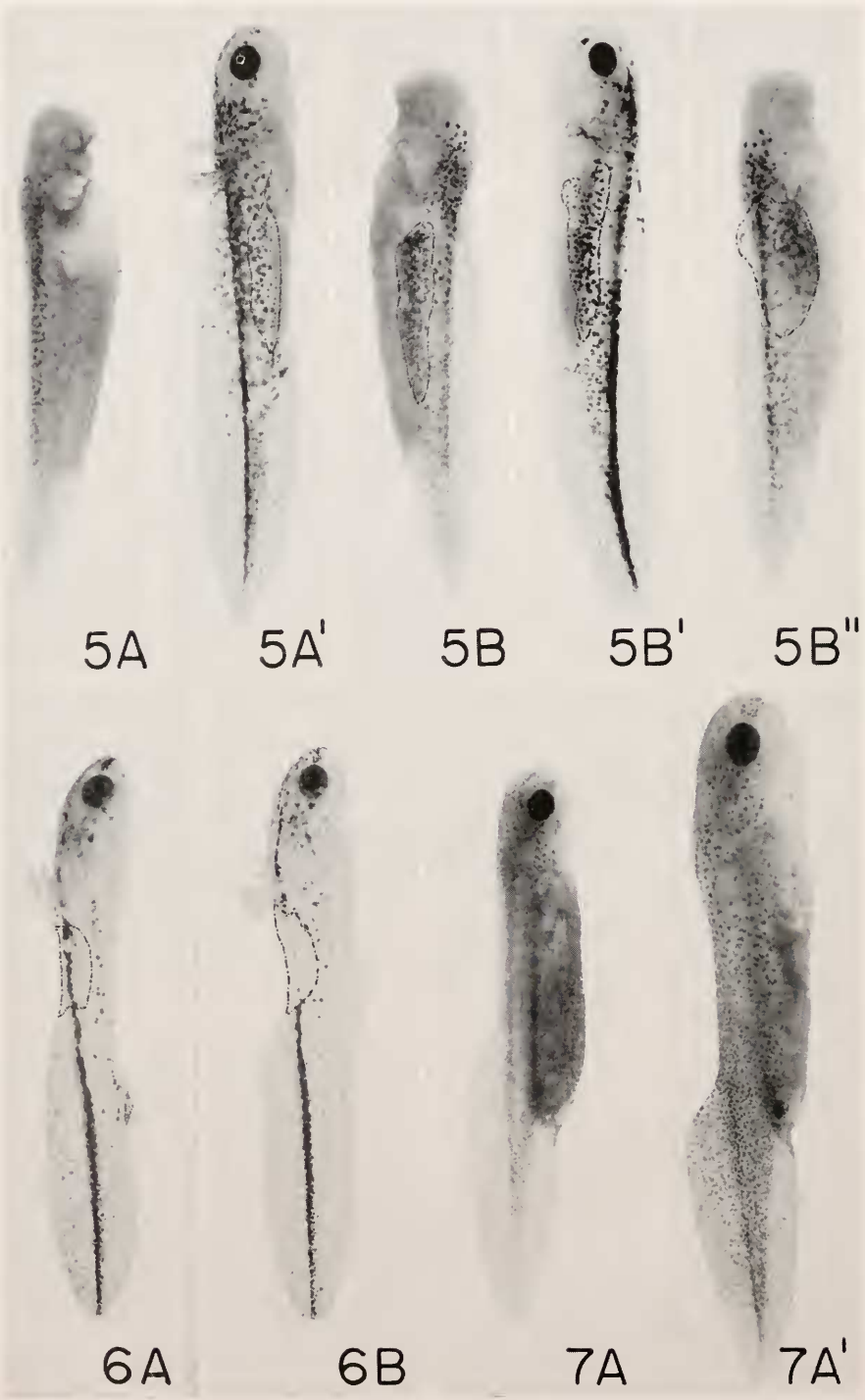
C. Intrinsic species differences in pro-pigment cell behavior

The question to receive immediate attention concerns the possibility that irrespective of the melanogenic character of surrounding tissues, the period and sequence of melanophore differentiation may be independently controlled by factors intrinsic to the pigment cells themselves. This possibility was suggested by differences in the normal manner of pigmentation in *T. torosus* and *T. rivularis* embryos, and by differences in the duration of the secondary delay in Triturus pigmentation on chimeric-chest embryos.

Series 7. Sequence of melanophore differentiation in species of Triturus and Amblystoma: The manner in which regions deprived of trunk neural crest became progressively invaded by melanophores from outlying regions provided the basis for distinguishing species differences in the time and sequence of melanophore differentiation. Trunk crest-less embryos were prepared by removing both trunk neural folds from Stage 16–17 neurulae. Following the operation, closure of the neural tube was essentially normal. Subsequent development was also normal ex-

[4] For the present, this interpretation has ignored a possible source of error which is currently being investigated, namely, the question of whether or not the time and degree of loss in melanogenic strength are similar in dorsal and ventral epidermis.

PLATE V



cept for the absence of neural crest derivatives and a dorsal fin in the region of extirpation. In all species used, the crest-less area remained free of differentiated pigment cells until Stage 39. However, the region eventually became populated by melanophores, and the sequence in which this was accomplished was taken as a measure of the inherent capacity of pro-pigment cells in each species to continue to differentiate over an extended developmental period.

Series 7a, 7b, and 7c dealt respectively with the development of trunk crest-less embryos of *T. rivularis* (Figs. 7A and 7A′), *A. mexicanum* (Figs. 7B and 7B′), and *A. punctatum* (Figs. 7C and 7C′). In these species, the invasion of crest-less areas by melanophores originating from levels anterior and posterior to the region of extirpation began at about Stage 40 and continued until the region was uniformly populated by pigment cells. Depending upon the anteroposterior extent of the crest-less area, pigmentation was completed at any time from pre-feeding to mid-larval stages of development. It was concluded that melanophore differentiation in these species can continue almost uninterruptedly over a major part of the developmental period.

Series 7d was concerned with trunk crest-less *T. torosus* embryos (Figs. 7D and 7D′), and provided results in sharp contrast to those obtained in the preceding series. The sequence of pigmentation in trunk crest-less animals closely paralleled that observed in normal *T. torosus* larvae in which two distinct populations of melanophores are recognized (*viz.*, the "primary melanophores" which appear between Stages 34 and 41 and re-aggregate into well defined dorsal bands that constitute the primary pigmentation of pre-feeding larvae, and the "secondary melanophores" which are smaller, less heavily pigmented cells that appear after the 18 to 20 mm. larval stage and permanently retain a random distribution on the fin and flanks. See Figures 8B and 8C, and Lehman, 1950). "Primary" *T. torosus* melanophores did not invade crest-less areas to any appreciable extent and retained the distribution achieved by Stage 39 (Fig. 7D). No additional pigmentation of crest-less regions occurred until the larvae reached a length of approximately 18

PLATE V

FIGURE 5A. Normal *T. torosus* embryo at Stage 36+, showing melanophores in the process of retracting toward the dorsal border of the somites.

FIGURE 5A′. *A. punctatum* → *T. torosus* chimeric-crest host bearing a flank graft of *A. punctatum* epidermis; site of graft indicated by broken line; figured at Stage 43.

FIGURE 5B. *A. mexicanum* → *T. torosus* chimeric-crest host bearing a flank graft of *A. mexicanum* epidermis; figured at Stage 37.

FIGURE 5B′. Same embryo shown in figure 5B, at Stage 41. Explanation in text.

FIGURE 5B″. Stage 37 embryo from the same series as figure 5B. The presence of darker host melanophores along the dorsal border of the myotomes, under grafted *A. mexicanum* epidermis that extended beyond the base of the fin, is shown.

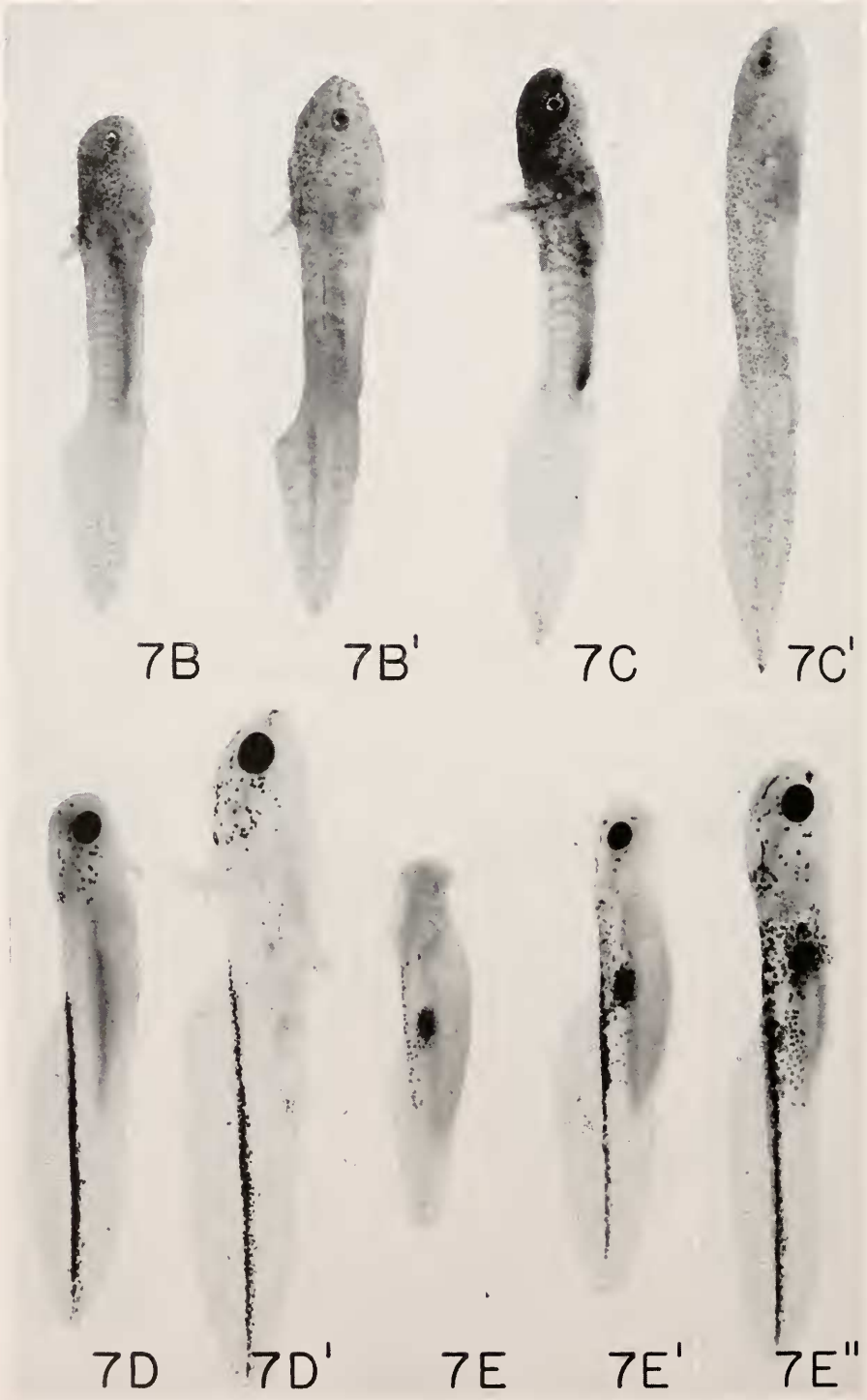
FIGURE 6A. An 18 mm. *T. torosus* larva in which Stage 13 + *T. torosus* belly epidermis was grafted mid-dorsally at Stage 28. Site of graft indicated by broken line.

FIGURE 6B. An 18 mm. *T. torosus* larva in which Stage 37 + *T. torosus* belly epidermis was grafted mid-dorsally at Stage 23. Explanation in text.

FIGURE 7A. Trunk crest-less *T. rivularis* embryo at Stage 40. Note deficiency in the dorsal fin and the absence of melanophores in the region of extirpation.

FIGURE 7A′. Embryo similar to that in figure 7A at Stage 45. The crest-less area has become populated by melanophores that have migrated into it from outlying region (original photograph by V. C. Twitty).

PLATE VI



mm. (Fig. 7D′). Thereafter, melanophore differentiation continued slowly through the remainder of the larval period. So far as could be determined on the bases of appearance and distribution, the gradual invasion of crest-less areas was accomplished entirely by "secondary" melanophores.

Series 7e demonstrated that *A. tigrinum* embryos, like those of *T. torosus,* have distinct "primary" and "secondary" generations of melanophores, which are recognizable on the bases of differences in time of pigmentation and definitive distribution. *A. tigrinum* embryos in neurula stages were not available and, instead, tail-bud embryos were used in the following manner. A graft of Stage 25 *A. tigrinum* neural crest was placed on the flank of Stage 32 *T. torosus* hosts which previously had been deprived of both trunk neural folds, so as to free the trunk region of host chromatophores. By the fourth post-operative day, donor melanophores were well differentiated on the trunk crest-less *T. torosus* hosts, which by this time had advanced to Stage 37. As can be seen in Figure 7E, the "primary" donor melanophores were aggregated along the mid-dorsal line or near the flank graft. Host cells are faintly visible in the tail. On the tenth post-operative day, at host Stage 40, "secondary" donor melanophores began to appear (Fig. 7E′). They were distinguishable from those that appeared earlier by permanently retaining a widespread distribution on the flanks. Their numbers gradually increased until both sides of the *T. torosus* hosts were densely populated (Fig. 7E″).

The above observations have the advantage of clearly showing that the discontinuous sequence of melanophore differentiation in *A. tigrinum* results from inherent differences in the developmental rates of primary and secondary melanophores, and is not due to the intervention of known environmental factors, as might be the case in *T. torosus* larvae. This conclusion is tenable, since the appearance of secondary *A. tigrinum* melanophores on Stage 40 + *T. torosus* hosts coincided in time with a known decline in the melanogenic strength of the host epidermis (see Series 6). So far, then, as the tissue environment of the secondary *A. tigrinum* pro-pigment cells was concerned, conditions were more favorable for pigmentation during host Stages 37 to 40, in which melanization failed to take place. This can be correlated with the fact that they are less "dependent" (DeLanney, 1941) upon environmental factors for pigmentation than are the cells of *T. torosus.* The interrupted sequence of *T. torosus* pigmentation also indicates that there are inherent differences between the primary and secondary melanophores in this species. However, it remains in question whether the differences concern

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PLATE VI

FIGURES 7B AND 7B′. Stage 41 and 16 mm. larval stage of a trunk crest-less *A. mexicanum* larva; pigmentation essentially as in figures 7A and 7A′.

FIGURES 7C AND 7C′. Stage 45 and 23 mm. larval stage of a trunk crest-less *A. punctatum* larva; pigmentation essentially as in figures 7A and 7A′.

FIGURES 7D AND 7D′. Stage 40 and 18 mm. larval stage of a trunk crest-less *T. torosus* larva. The region of extirpation remained essentially free of melanophores until shortly after this period. A few secondary melanophores are visible at the base of the fore limb in figure 7D′.

FIGURE 7E. A trunk crest-less *T. torosus* host bearing an *A. tigrinum* neural crest graft on the flank; figured 7 days after operation. Explanation in text.

FIGURE 7E′. The same embryo shown in figure 7E, 13 days after the operation.

FIGURE 7E″. The same embryo shown in figures 7E and 7E′, 21 days after the operation.

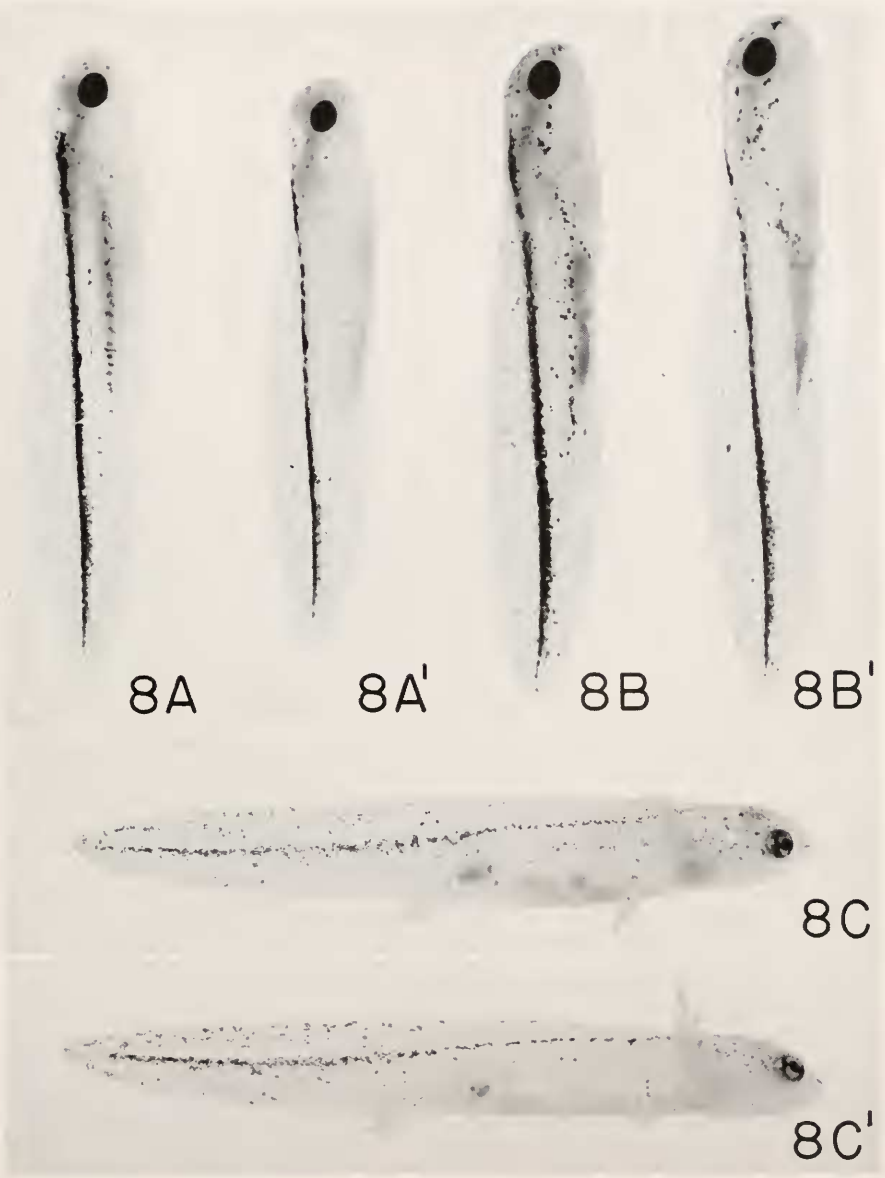
rates of development or differences in sensitivity to environmental factors such as melanogenic materials or larval hormones. Unfortunately, the evidence on hand does not provide an answer to this question. Possibly both an intrinsic limitation of the period of differentiation, and the declining melanogenic strength of the epidermis contribute to the failure of primary *T. torosus* melanophores to develop except during Stages 32 and 41. If this were indeed the case, the temporal coincidence in the action of intrinsic and environmental factors would provide an example of the well known principle of "double assurance" (Braus, 1906, and Spemann, 1938, pp. 92–97) ; that is, either factor by itself might be able to prevent the differentiation of primary *T. torosus* melanophores after Stage 41.

Series 8. Unilateral extirpation of trunk neural folds from T. rivularis and T. torosus Stage 17 neurulae: The manner in which crest-less areas became pigmented in embryos of *T. rivularis* (Series 7a) and *T. torosus* (Series 7d) suggested the presence of yet another difference between these species. If one considers only those pigment cells which appear during pre-feeding stages, the impression is given that the capacity of neural crest to supply melanophores in excess of those normally differentiating is much greater in *T. rivularis* than in *T. torosus*. This possibility was examined by subjecting Stage 17 neurulae of both species to unilateral extirpation of one entire trunk neural fold. The operation did not interfere with the closure of the neural tube and by this means it was possible to reduce the amount of tissue comprising the neural crest to one-half that found in normal embryos. Embryos treated in this manner will hereafter be referred to as "half-crest"embryos or larvae.

When the pre-feeding larvae were later examined for deficiencies in the melanophore population, it was found that half-crest *T. rivularis* larvae were indistinguishable from normal animals with regard to number and distribution of pigment cells. Therefore, since one *T. rivularis* neural fold can give rise to a full complement of pigment cells, it is concluded that the regulatory capacity of *T. rivularis* neural crest is at least two times greater than the normal expression of its ability to produce pigment cells.

The number of *T. torosus* melanophores on half-crest embryos was conspicuously lower than that on normal larvae from the same egg clutch. In Figures 8A and 8A′, one will note that the dorsal band on the half-crest embryo is narrower than the band on the control. This is a direct reflection of the smaller number of primary melanophores present. The experimental animals moreover differed from the controls by lacking melanophores at the yolk border. The absence of melanophores at the yolk border can be explained by the fact that the distance travelled by a given pigment cell is roughly proportional to the number and proximity of other pigment cells (Twitty and Niu, 1948). Hence, with fewer propigment cells to exhibit mutual antagonisms, migration probably failed to proceed as far ventrally as the yolk border and all the cells were later withdrawn from the flanks in the formation of the dorsal bands. Cell counts made on 16 to 17 mm. larvae while the melanophores were in the contracted phase revealed that the number of primary melanophores in the dorsal bands of half-crest embryos was 30 to 40 per cent lower than the number found in normal larvae (Figs. 8B and 8B′). Thus, even though the single neural fold contributed more primary melanophores than it would have in a control embryo, the regulatory capacity of *T. torosus* neural

PLATE VII



FIGURES 8A, 8B, AND 8C. A normal *T. torosus* at pre-feeding larval Stage 41, and at 17 and 30 mm. larval stages respectively, showing the gradual appearance of secondary melanophores.

FIGURES 8A′, 8B′, AND 8C′. A half-crest *T. torosus* larva at corresponding stages, showing a reduction in the number of primary melanophores in the dorsal band.

crest presumably does not exceed 1.2 to 1.4 times the number of primary pro-pigment cells which normally differentiate. A limitation of this sort apparently does not apply to the quantity of secondary melanophores produced. The differentiation of these cells in half-crest larvae lagged only slightly behind that observed in controls (Figs. 8C and 8C').

Discussion

The experimental analysis of amphibian pigmentation has at present progressed to a point that permits an array of interacting factors to be recognized as mediators in the final expression of chromatophore development (see review by Twitty, 1949). By acknowledging the complexity of the overall problem of pigmentation, it was possible to avoid the initial error of expecting that a single factor such as growth rate, age, or species differences might alone be responsible for the phenomenon of pigment suppression with which this paper is concerned. The following factors have been identified as collective determinants in pro-pigment cell differentiation: 1) the ability of partially differentiated melanophores to be more effective than mature cells in repelling invasion by younger pro-pigment cells, 2) genetic, regional, and temporal differences in the capacity of embryonic ectoderm and mesoderm to promote melanin synthesis, 3) a possible competition between chromatophores for terrain and melanogenic substances, and 4) species differences in the behavior of chromatophores with respect to, a) their dependence upon the environment for melanogenic substances, b) the sequence of melanophore differentiation during development, and c) the regulatory capacity of the neural crest to produce more than the normal number of pigment cells. It is apparent that as these conditions are altered by transplantation between embryos of different age or genetic constitution, there will be modifications in the degree to which the prospective potency of pro-pigment cells is realized. The degrees of pigment suppression obtained thus may vary from complete (in which the concerted action of extrinsic factors works to prevent the differentiation of one group of pro-pigment cells) to very indifferent manifestations of this phenomenon. It is necessary to realize that temporal changes continually alter not only the cellular environment but also the intrinsic capacities of the cells. Synchronized timing is therefore of cardinal importance in pigment suppression, since the results in a given instance are colored by the sequence with which each of the above factors comes into play, and independently changes as development proceeds.

Before proceeding to the interpretation of suppression in specific chimeric-crest combinations, it should be pointed out that the major contrast between the melanophores of *T. rivularis* and *T. torosus* is provided by a comparison of the cells of *T. rivularis* with the "*primary*" melanophores of *T. torosus*. If, instead, only the "*secondary*" *T. torosus* cells were considered, one would find that the similarities far outweigh the differences. For example, both *T. rivularis* and secondary *T. torosus* melanophores are characterized, 1) by being relatively independent of the environment for melanogenic substances, 2) by permanently retaining a random, widespread distribution, 3) by being highly regulatory with regard to numbers, and 4) by exhibiting continuous differentiation of new cells over much of the developmental period. These traits probably should be considered primitive chromatophore characteristics. Primary *T. torosus* melanophores, with their sen-

sitivity to melanogen fluctuations, their tendency to undergo re-aggregation, and their limitations in number and time of differentiation, suggest that here one is dealing with a highly specialized type of pigment cell (see Twitty, 1945, pp. 173–174).

The remainder of the discussion will attempt to define the probable roles of intrinsic and environmental factors influencing melanophore differentiation in the Amblystoma—Triturus chimeric-crest experiments (Lehman, 1950), which provided the point of departure for the present study.

Analysis of suppression in A. punctatum → T. rivularis chimeric-crest embryos: The suppression of *T. rivularis* melanophores was not striking in this combination and may be viewed simply as delayed pigmentation in which migration and melanogenesis were temporarily held in abeyance by the antagonistic action (see Series 1, 2, and 3) of rapidly developing *A. punctatum* cells. *T. rivularis* melanophores began to appear in flank areas almost as soon as *A. punctatum* melanophores had completed their differentiation at Stage 40 +. Until shortly before this time, they presumably were immobilized in the vicinity of the mid-dorsal line. The disparity between the developmental rates of the two species was apparently too slight to provide more than a transitory impediment to the outgrowth and differentiation of *T. rivularis* cells which have no sharply restricted period during which pigmentation must occur (Series 7a). The differentiation of *T. rivularis* pigment cells at a time when the epidermis had probably become weakly melanogenic (Series 6) can be correlated with the fact (DeLanney, 1941) that their differentiation is relatively "independent" of environmental factors, whereas *T. torosus* melanophores are very sensitive to variations in the melanogenic character of the surrounding tissues. Thus, a decline in the ability of ageing epidermis to foster pigmentation need not interfere appreciably with *T. rivularis* pigmentation, but still might be very effective in preventing the differentiation of *T. torosus* melanophores during pre-feeding larval stages.

Analysis of suppression in A. mexicanum → T. rivularis chimeric-crest embryos: The primary difference between this and the preceding series centered around the failure of *T. rivularis* melanophores to appear on the flank until approximately Stage 44 +. It is surmised that after Stage 39 (when donor *A. mexicanum* melanophores appeared to be fully differentiated and host cells were appearing in the dorsal fin), latent melanophores of both species were present on the flanks and thereafter competed, perhaps on equal footing, for the limited remaining terrain. Some unidentified intrinsic property, such as a more rapid division rate or stronger intercellular antagonisms, may have enabled *A. mexicanum* melanophores to "saturate" the terrain more completely than those of *A. punctatum* in the preceding series. As a result, there may have been less available space and sharper competition for melanogenic materials, which could account for the slight secondary delay in the appearance of *T. rivularis* melanophores.

Analysis of suppression in A. punctatum → T. torosus chimeric-crest embryos: The lowest level of pigment suppression was encountered in this combination. This is correlated with the fact that during the critical period between Stages 32 and 38, the developmental rates of the two species are very nearly equal (see Leh-

man, 1950, Figure 3). Therefore, it might be expected that the pro-pigment cells of both neural crest components would initiate migration and undergo pigmentation almost simultaneously. Under such conditions, intercellular antagonism between developing chromatophores would be responsible for the general spreading and dispersion of *T. torosus* and *A. punctatum* pro-pigment cells alike. The number of *T. torosus* melanophores which differentiated was approximately one-third the number appearing on normal embryos. This is not surprising when one considers that the removal of one *T. torosus* neural fold would, of itself, result in a 30 to 40 per cent reduction in the number of primary *T. torosus* melanophores developing (Series 8), irrespective of whether or not a fold from another species were added. Consequently, the suppression of primary melanophores in all *T. torosus* chimeric-crest larvae would appear to be more pronounced than is actually warranted. In *T. rivularis* combinations, however, the effect of eliminating one neural fold can, for all practical purposes, be discounted, since a single neural fold is capable of supplying a normal complement of pigment cells. The final factor to consider is the melanogenic strength of surrounding tissues which restricts the density of the melanophore population. Faced with an environmental limitation of this sort, it follows that donor and host cells of nearly equal developmental rates will act reciprocally upon each other in competing for space and melanogenic substances, thereby establishing a balance that holds in check the capacity of both donor and host cells to undergo melanization.

Analysis of suppression in A. mexicanum → T. torosus chimeric-crest embryos: Maximal inhibition of primary *T. torosus* melanophores was obtained in this combination and probably resulted from the concerted action of several factors. It was suggested "that the most favorable conditions for pigment inhibition are realized (as in *A. mexicanum → T. torosus* combinations) when the difference in rate of pigmentation is of such magnitude as to permit rapidly developing donor chromatophores to appear initially at approximately host Stage 31–32" (Lehman, 1950, p. 448). It now appears that this relationship obtains because, at the time at which *T. torosus* pro-pigment cells would normally begin to migrate from the neural crest at Stages 28 to 31, *A. mexicanum* pigment cells had already appropriated all available areas. More important, the *A. mexicanum* cells were at the stage during which the capacity to prevent invasion was at a peak (Series 1, 2, and 3). Consequently, during the initial migratory period *T. torosus* melanophores were probably unable to gain access to the flank terrain (Series 5b). *A. mexicanum* melanophores were visible on the flanks by Stage 32 and new cells were evidently able to differentiate continuously during much of the larval period (Series 7b). After Stage 41, the intrinsic inability of primary *T. torosus* melanophores to become pigmented (Series 7d), or the temporal decline in the melanogenic strength of the epidermis (Series 6), would be sufficient to prevent the appearance of primary host cells. Competition between donor and host cells was probably not an important factor in this combination. The only region in which it probably occurred was along both sides of the mid-dorsal line where some intermingling of donor and host cells very likely took place prior to Stage 41. The actual fate of latent primary *T. torosus* cells is as yet undetermined. However, it is likely that after failing to achieve their prospective fate, they enter into some other channel of neural crest development, *e.g.*, secondary melanophores, neurons, mesenchyme, etc.

Analysis of suppression in A. tigrinum → T. torosus chimeric-crest embryos: The degree to which *T. torosus* primary melanophores were suppressed by *A. tigrinum* cells was less than that encountered in the preceding series, even though *A. tigrinum* embryos developed appreciably more rapidly than those of *A. mexicanum*. In order to explain this paradox, it is necessary to make the assumption that *A. tigrinum* melanophores became fully differentiated before host Stage 41. That is to say, the donor cells must have lost the ability to repel host cells and were incapable of lowering chromogen concentration to sub-threshold levels while the primary *T. torosus* pro-pigment cells were still able to differentiate and while the epidermis was able to promote their pigmentation. It has been shown that the melanophores of *A. tigrinum* have an interrupted sequence of pigmentation (Series 7e), and that after the primary *A. tigrinum* melanophores became well pigmented, a period of five to six days elapsed before additional cells began to appear. Since the primary donor melanophores on *A. tigrinum → T. torosus* chimeric-crest embryos were fully differentiated by host Stage 35 (Lehman, 1950), the secondary donor cells probably began to appear at approximately host Stage 38. The few *T. torosus* melanophores which made an appearance probably did so during the interval between the two successive periods of donor differentiation. An argument in support of this view is provided by the observation that *T. torosus* melanophores were not seen until Stage 38. This is taken as evidence that the migration and pigmentation of these cells had temporarily been delayed until primary donor melanophores were in an advanced stage of development. However, even after Stage 38, the number of *T. torosus* melanophores which became pigmented was small. This perhaps should be expected when one recalls that a relatively short period remained after Stage 38, during which the epidermal strength was sufficient to permit pigmentation and the primary *T. torosus* cells were still able to respond. The final factor is the role played by secondary *A. tigrinum* melanophores. They very likely began to emerge simultaneously with the primary host cells at Stage 38, and active competition between them would tend to reduce still further the number of host cells which otherwise might have appeared.

It is appropriate, therefore, to point out that in the case of *A. tigrinum* and *T. torosus* embryos in which there are distinct primary and secondary melanophore generations, a condition exists in the normal embryo which is somewhat comparable to that in chimeric-crest embryos. Although experimental evidence is lacking, it is possible that in *A. tigrinum*, immature primary melanophores may prolong a normal slight delay in the migration and pigmentation of secondary cells. The prospective secondary melanophores of *T. torosus* may also be able to migrate onto the flanks *only* after the primary cells have completed their differentiation; however, owing to a melanogen deficiency, they remain unpigmented until some other factor (probably hormonal) comes into play and permits their gradual differentiation during larval stages. In the embryos of *A. mexicanum*, *A. punctatum* and *T. rivularis*, the sequence of chromatophore differentiation is apparently continuous, and no visible distinction can be made between early and later generations of pigment cells. Nevertheless, even in these species the initial population of melanophores may impose a partial barrier that retards the outgrowth and pigmentation of cells following after them. Owing to its regulatory capacity, the neural crest of most species is capable of providing many more chromatophores than normally become pigmented. The limiting factor in their development is established by the epidermis and mesoderm, which

govern the number of melanophores than can appear in a given area. One might consider that the pigment cells which do differentiate in normal development are those which have successfully competed for the limited facilities of the environmental terrain and thereby have, in effect, "suppressed" other cells in the same area. The prospective melanophores eliminated in the competition for space and melanogenic materials may eventually become pigmented, or be consigned to other channels of neural crest development. In either event, they very likely share the fate, whatever it may be, of cells similarly "suppressed" in chimeric-crest embryos.

Viewed in this light, pigment suppression in chimeric-crest embryos may be merely an accentuated expression of processes operating in normal development. That is, the grafted Amblystoma cells may simply appropriate the role normally played by the initial complement of Triturus melanophores, with the result that an increased percentage of host cells is forced to accept the status and fate of unpigmented prospective melanophores.

Summary

The present study was undertaken for the purpose of clarifying the developmental mechanics operating in the phenomenon of pigment suppression in salamanders. The experiments include the transplantation of epidermis and the explantation, transplantation and extirpation of neural folds and neural crest in five species of salamanders. These species, listed in the order of increasing rates of development, are: *Triturus rivularis*, *T. torosus*, *Amblystoma punctatum*, *A. mexicanum* and *A. tigrinum*.

1. By culturing two fragments of neural crest of different age in single drops of culture medium, it was demonstrated that incompletely differentiated melanophores offer a more effective barrier to the migration of young pro-pigment cells than do the cells of fully mature outgrowths.

2. The same relationship between intermediate stage of differentiation and maximal ability to prevent invasion by younger cells was demonstrated *in vivo* by transplanting young *A. punctatum* neural crest on the flanks of *T. rivularis* embryos of graded age.

3. It was found that the degree of pigment suppression obtained in "standard" chimeric-crest embryos (unilateral orthotopic grafts of trunk neural folds) could be reduced either by prolonged chilling of the embryos following the operation, or by using donor and host neurulae of different developmental age. It is assumed that in both instances, the normal difference in donor and host developmental rates was altered in such a manner that an optimal intermediate disparity was not realized.

4. Chimeric-crest taken from *A. mexicanum* → *T. torosus* embryos (a combination in which suppression is maximally expressed) and explanted in peritoneal fluid gave mixed outgrowths of *A. mexicanum* and *T. torosus* cells, both types of which eventually became pigmented. This result is noteworthy, inasmuch as had they developed *in vivo*, the *T. torosus* cells undoubtedly would have been suppressed.

5. Amblystoma epidermis which is "strongly melanogenic" was grafted on the flanks of chimeric-crest hosts as a means of testing for the presence of unpigmented *T. torosus* cells on the flanks. *T. torosus* melanophores did differentiate under epidermal flank grafts on *A. punctatum* → *T. torosus* hosts (a combination exhibiting a

low degree of suppression), but they failed to appear in *A. mexicanum* → *T. torosus* hosts except under special circumstances. It is concluded that *A. mexicanum* cells had prevented the outgrowth and differentiation of *T. torosus* pro-pigment cells.

6. Homoplastic transplantations of belly epidermis from *T. torosus* donors of graded age mid-dorsally onto tail-bud hosts revealed that between Stages 38 and 40 +, *T. torosus* epidermis loses most of its capacity to promote pigmentation in Triturus melanophores. It is suggested that this may be responsible for secondarily delaying the differentiation of *T. torosus* pro-pigment cells after the donor cells are well differentiated on chimeric-crest hosts.

7. Observations on the manner in which trunk regions deprived of neural crest became invaded by melanophores indicate that melanophore differentiation is essentially a continuous process in *T. rivularis*, *A. mexicanum* and *A. punctatum* embryos. In *T. torosus* and *A. tigrinum* embryos, the sequence of pigmentation was found to be interrupted, and consisted of distinct "primary" and "secondary" generations of melanophores.

8. It was noted that the removal of one trunk neural fold from *T. rivularis* embryos had no observable effect upon the number of pigment cells that later appeared. In similarly treated *T. torosus* embryos, however, this resulted in a 30 to 40 per cent reduction in the number of cells normally appearing.

The results of previous chimeric-crest experiments (Lehman, 1950) are analyzed in the light of the above findings.

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